Assessing the Frequency and Consequences of *Salmonella enteritidis* Deposition on the Egg Yolk Membrane

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ABSTRACT The site of deposition of *Salmonella enteritidis* in eggs could influence the extent to which this pathogen multiplies before refrigeration achieves growth-inhibiting internal temperatures. The first part of this study sought to determine whether *S. enteritidis* inoculated onto the exterior (vitelline) membrane surface of egg yolks was able to penetrate into and multiply within the yolk contents. When 10^2 cfu of *S. enteritidis* was inoculated onto the exterior surface of intact egg yolks, multiplication within the interior yolk contents occurred in 10% of samples after 6 h of incubation and in 75% of samples after 24 h at 25 C (reaching mean levels of about 10^4 cfu/mL) but in only 20% of samples incubated for 72 h at 15 C. The second part of this study applied an oral infection model in laying hens to establish the relative proportions of contaminated eggs in which *S. enteritidis* deposition was associated with the yolk membrane or was found inside the yolk contents. Although approximately 4.3% of egg yolks were positive for *S. enteritidis* when both yolk contents and membranes were sampled, only about 0.5% of samples of yolk contents (without membranes) were positive. Although deposition of *S. enteritidis* within egg yolks appears to occur infrequently, rapid refrigeration of eggs is necessary to prevent the penetration of *S. enteritidis* into and multiplication within egg yolks.

(*Key words*: *Salmonella enteritidis*, egg, deposition, yolk membrane, refrigeration)

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INTRODUCTION

The transmission of *Salmonella enteritidis* infections to humans by contaminated eggs has been a prominent food safety issue throughout much of the world for more than a decade (Angulo and Swerdlow, 1999; Centers for Disease Control, 2000). Diverse risk reduction strategies have been recommended and incorporated into regional and national microbial quality assurance programs for eggs (Hogue et al., 1998). Prospective targets for *S. enteritidis* control programs exist along the entire egg production and utilization continuum (President's Council on Food Safety, 1999). The potential effectiveness of many such control measures depends on having a thorough understanding of the underlying biological processes that result in the internal contamination of eggs laid by systemically infected hens. One important instance of this generalization concerns the manner in which the spatial distribution of *S. enteritidis* deposition inside eggs affects the outcome of efforts to apply refrigeration for restricting bacterial multiplication.

Contaminated eggs are apparently produced very infrequently by commercial laying flocks in the United States. Sampling studies conducted in naturally infected flocks have reported egg contamination frequencies below 0.03% (Kinde et al., 1996; Schlosser et al., 1999) and the overall national incidence of *S. enteritidis* in eggs has been estimated at 0.005% (Ebel and Schlosser, 2000). Moreover, most available evidence suggests that egg contamination by *S. enteritidis* typically involves relatively small initial numbers of bacterial cells (Humphrey et al., 1989, 1991; Gast and Beard, 1992; Gast and Holt, 2000b). Infected hens can deposit *S. enteritidis* in the yolk or albumen of developing eggs (Humphrey et al., 1989, 1991; Gast and Beard, 1990; Bichler et al., 1996; Gast and Holt, 2000b), perhaps depending on which regions of the reproductive tract are colonized by the pathogen. Although very little bacterial multiplication occurs in egg albumen, *S. enteritidis* can persist there at supportive temperatures (Lock and Board, 1992; Baron et al., 1997; Gast and Holt, 2000a). However, egg yolk can support rapid and extensive growth of *S. enteritidis*, especially at storage temperatures above 20 C (Bradshaw et al., 1990; Clay and Board, 1991; Humphrey and Whitehead, 1993; Braun and Fehlhaber, 1995; Gast and Holt, 2000a).

Refrigeration of eggs during transportation and storage at an ambient temperature of 7 C has been proposed and instituted as a means of preventing small initial numbers of *S. enteritidis* contaminants from multiplying to levels

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more likely to cause human illness (USDA, 1998; President's Council on Food Safety, 1999). However, conventional refrigeration technologies may require several days to lower internal egg temperatures sufficiently to restrict bacterial growth (Curtis et al., 1995; Thompson et al., 2000). Although this cooling period has few adverse consequences if *S. enteritidis* contaminants remain in the albumen, the initial deposition of *S. enteritidis* within yolks or subsequent bacterial penetration into yolks (Humphrey and Whitehead, 1993; Braun and Fehlhaber, 1995; Gast and Holt, 2000a) could allow multiplication of *S. enteritidis* to reach more dangerous levels during the early stages of refrigeration. Accordingly, determining where *S. enteritidis* is deposited in eggs and the extent to which penetration to reach the nutrient-rich yolk contents occurs are critical issues for developing and applying effective egg refrigeration standards. Prior work has demonstrated that *S. enteritidis* contamination can be associated with egg yolks, but these studies have not conclusively established whether *S. enteritidis* is more often deposited inside yolks or on the exterior (vitelline) membrane surrounding yolks. The objectives of the present study were to determine the frequency at which *S. enteritidis* contaminants inoculated onto the yolk membrane penetrated to reach the interior yolk contents and whether experimentally infected hens more often deposited *S. enteritidis* on the yolk membrane or inside the yolk contents.

MATERIALS AND METHODS

Preparation of S. enteritidis *Cultures*

Frozen storage beads containing phage type 8 or phage type 13a strains of *S. enteritidis* (for inoculation of egg contents samples and laying hens, respectively) were resuscitated and prepared for use during two cycles of inoculation for 24 h at 37 C in tryptone soya broth.[2] This culture was centrifuged for 10 min at 3,000 × *g* to concentrate cells, washed with 0.85% saline, centrifuged again, and then resuspended in saline. After the cell density of the resuspended culture was estimated by determining its optical density at 600 nm, further dilution in saline produced cultures containing the desired final target cell densities (confirmed by subsequent plate counts).

Preparation, Inoculation, and Incubation of Egg Content Samples

In each of two replicate trials, freshly collected eggs from our laboratory's specific-pathogen-free flock of Single Comb White Leghorn chickens were aseptically broken, and their contents (yolk and albumen) were separated and transferred to sterile plastic beakers. Two types of samples were prepared and inoculated with *S. enteritidis*. Each yolk contents sample consisted of an entire yolk that was inoculated internally. Each yolk membrane sample consisted of an entire yolk that was inoculated on the exterior surface of its vitelline membrane and held at room temperature for 5 min, after which the albumen from a single egg was poured gently into the beaker to surround the yolk. Yolk membrane samples provided an assessment of the ability of *S. enteritidis* to penetrate into and multiply within the yolk contents when initially deposited on the outside surface of the yolk. Yolk content samples simulated the level of bacterial multiplication that would be observed if similar numbers of *S. enteritidis* were initially deposited inside yolks. Each sample was inoculated with a 0.1-mL dose containing 100 cfu of *S. enteritidis*. Sixty samples of each type were prepared per trial; 40 of these were incubated at 15 C after inoculation, and 20 were incubated at 25 C.

Enumeration of S. enteritidis *in Egg Content Samples after Incubation*

After 6 and 24 h of incubation, 20 samples of each type (10 per incubation temperature) were removed and cultured to enumerate *S. enteritidis*. Ten additional samples of each type were similarly removed from 15 C incubation and tested after 48 h and 10 more were tested after 72 h. For culturing, the yolk from each sample was transferred to a sterile petri dish. A small area of the yolk membrane was seared with a flame-heated steel spatula to destroy any bacteria present in that region. A sterile syringe was then inserted through the seared area to remove 5 mL of interior yolk contents (free of membrane). The level of *S. enteritidis* contamination was determined by making serial 10-fold dilutions of each yolk contents sample in saline and spreading aliquots of each dilution onto plates of brilliant green agar[3] supplemented with 0.02 mg/mL novobiocin.[4] The agar plates were incubated for 24 h at 37 C, and *S. enteritidis* colonies were identified (Waltman et al., 1998) and counted. The detection threshold of this procedure was 100 cfu/ml.

Experimental Infection of Laying Hens

In each of two replicate trials, 28 laying hens obtained from our laboratory's specific-pathogen-free flock of Single Comb White Leghorn chickens were housed in a disease-containment isolation facility. The hens (26 and 32 wk old at the beginning of the first and second trials, respectively) were housed individually in laying cages and provided with water and pelleted feed (16.7% CP, 2,968 kcal ME/kg, 2.9% Ca, 0.39% P) ad libitum. Each hen was inoculated orally with a 1.0-mL dose containing 2.0×10^9 cfu of *S. enteritidis*.

Isolation of S. enteritidis *on and Within Egg Yolks from Experimentally Infected Hens*

All eggs laid between 4 and 22 d after inoculation were collected for testing. This sampling interval surrounded

[2]Unipath Co., Oxoid Division, Ogdensburg, NY 13669.
[3]Difco Laboratories, Detroit, MI 48232.
[4]Sigma Chemical Co., St. Louis, MO 63178-9916.

the peak time for egg contamination (at around 11 to 12 d postinoculation) as determined in similar previous infection studies (Gast and Beard, 1990). Eggs were collected and cultured daily on weekdays. Eggs collected on weekends were held for up to 48 h at 7.2 C before culturing. Eggshell surfaces were disinfected by dipping for 5 s in 70% ethanol, and the shells were broken against sterile foil strips to release the contents. Yolks were aseptically separated and transferred to sterile petri dishes. As described above, the surface of each yolk membrane was seared with a flame-heated spatula, and a 5-mL samples of yolk contents (free of membrane) was removed (from a typical total yolk volume of approximately 12 mL). The yolk contents sample and the remainder of the yolk (including contents and membrane) were then each transferred to 50-mL centrifuge tubes containing 25 mL of tryptone soya broth supplemented with 35 mg/L ferrous sulfate, were mixed by vigorous shaking for 5 s, and were incubated for 24 h at 37 C. A 1-mL portion of each incubated sample was then transferred to 9 mL of Rappaport Vassiliadis broth[2] and again incubated for 24 h at 37 C. An inoculating loop was then used to streak aliquots of each culture onto plates of brilliant green agar containing 0.02 mg/mL novobiocin, which were incubated for 24 h at 37 C. The identity of prospective *S. enteritidis* colonies was confirmed biochemically and serologically (Waltman et al., 1998).

Statistical Analysis

Significant differences ($P < 0.05$) between trials or sample types in the mean frequency of recovery of *S. enteritidis* were determined by applying Fisher's exact test to data organized into 2×2 contingency tables with Instat biostatistics software.[5] Significant differences ($P < 0.05$) between trials or sample types in the mean level of recovery of *S. enteritidis* were similarly evaluated using an unpaired *t*-test. Because no significant variation was observed between replicate trials, the results were combined for analysis.

RESULTS

Isolation and Enumeration of S. enteritidis *from Inoculated Egg Yolks*

Following inoculation onto the exterior surface of yolk membranes at initial levels of approximately 8 cfu/mL of yolk, *S. enteritidis* was not isolated from the yolk contents after 6 and 24 h of incubation at 15 C (Figure 1a). Penetration of *S. enteritidis* through the yolk membrane to reach the interior contents (and multiplication to reach the detection threshold of 100 cfu/mL) was evident in 5% of samples after 48 h and in 20% of samples after 72 h at 15 C. The mean $\log_{10}$ levels of *S. enteritidis* in yolk contents from these samples were 0.22 cfu/mL at 48 h and 0.95 cfu/mL at 72 h (Figure 1b). When similar initial numbers of *S. enteritidis* were introduced directly into yolk contents, 55% of samples were detected as positive after 6 h of incubation at 15 C, and 100% were positive by 24 h (Figure 1a). Direct inoculation of yolk contents with *S. enteritidis* resulted in mean $\log_{10}$ levels of 3.76 cfu/mL and 6.97 cfu/mL after 48 h and 72 h, respectively (Figure 1b).

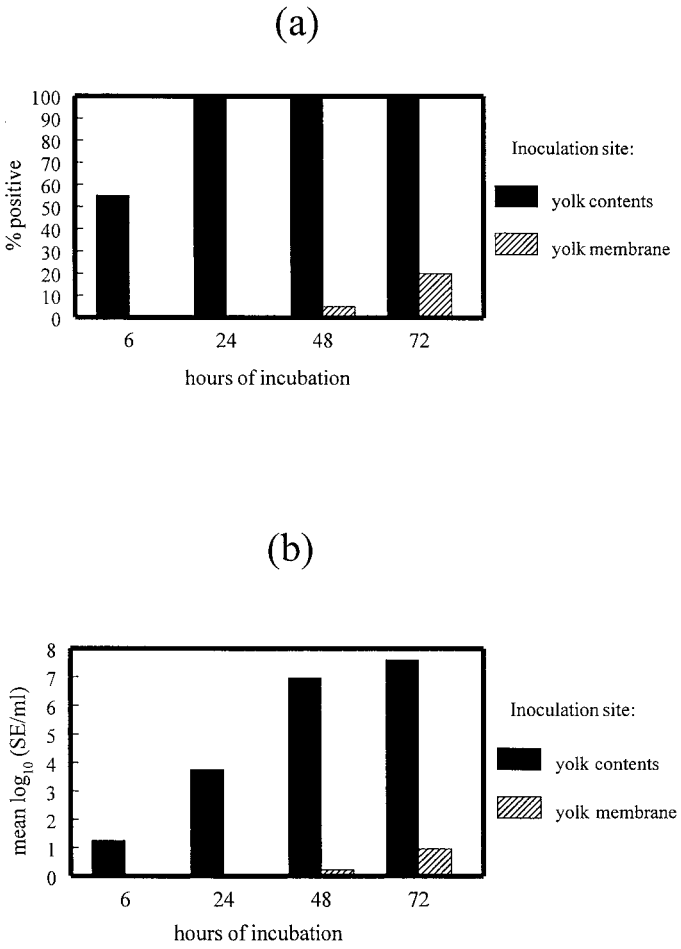

FIGURE 1. Isolation and enumeration of *Salmonella enteritidis* (SE) from the contents of inoculated egg yolk samples after incubation at 15 C. Individual yolks were inoculated by injecting 100 cfu of SE (approximately 8 cfu/mL of yolk) directly into the interior contents or by pipetting a similar dose of SE onto the exterior membrane surface and then adding back the albumen (n = 20 for each sample type). (a) Frequency of isolation of SE from the interior contents of incubated yolks; (b) enumeration of SE in the interior contents of incubated yolks.

After inoculation onto the exterior yolk membrane surface and incubation at 25 C, *S. enteritidis* was recovered from the interior contents of 10% of yolks after 6 h and from 75% after 24 h (Figure 2a). Mean $\log_{10}$ levels of *S. enteritidis* in the contents of these yolks reached 0.23 cfu/mL at 6 h and 3.97 cfu/mL at 24 h (Figure 2b). All samples inoculated directly into the yolk contents and incubated at 25 C were positive for *S. enteritidis* by 6 h (Figure 2a), at mean $\log_{10}$ levels of 4.34 cfu/mL at 6 h and 8.40 cfu/mL at 24 h (Figure 2b). At all temperatures and incubation

[5]GraphPad Software, San Diego, CA 92121.

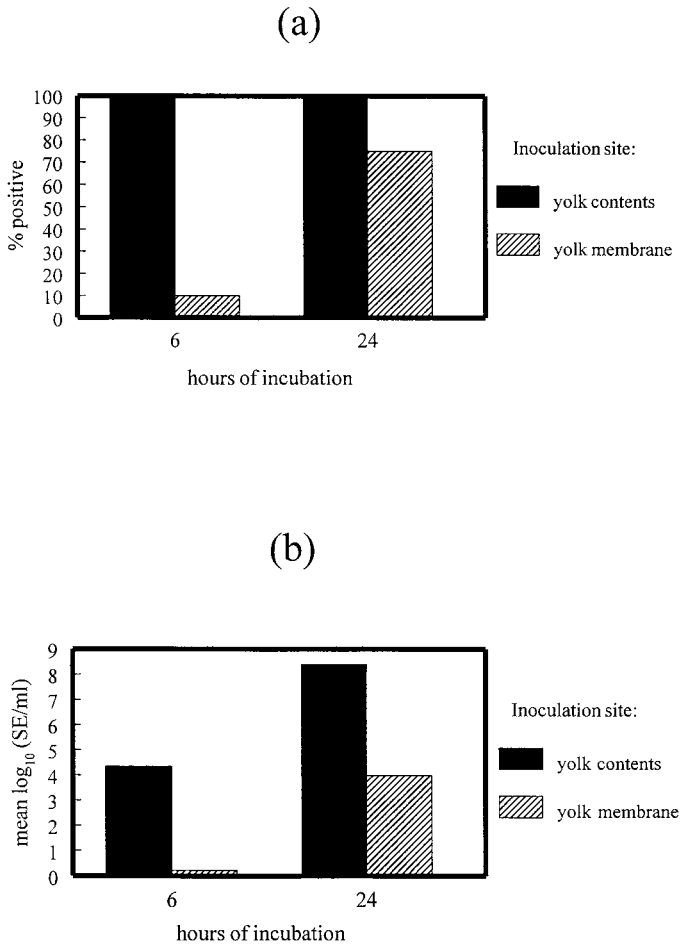

FIGURE 2. Isolation and enumeration of *Salmonella enteritidis* (SE) from the contents of inoculated egg yolk samples after incubation at 25 C. Individual yolks were inoculated by injecting 100 cfu of SE (approximately 8 cfu/mL of yolk) directly into the interior contents or by pipetting a similar dose of SE onto the exterior membrane surface and then adding back the albumen (n = 20 for each sample type). (a) Frequency of isolation of SE from the interior contents of incubated yolks; (b) enumeration of SE in the interior contents of incubated yolks.

intervals, the frequencies and levels of *S. enteritidis* recovery from yolk contents samples were significantly ($P < 0.0015$) greater than from yolk membrane samples.

Isolation of S. enteritidis *from Egg Yolks Laid by Experimentally Infected Hens*

In Trial 1, *S. enteritidis* was recovered from only 2 of 350 eggs (0.57%) of infected hens when the interior yolk contents were cultured without the surrounding yolk membrane, but from 13 of these same 350 eggs (3.71%) when both the yolk contents and membrane were tested (Figure 3). In Trial 2, only 1 of 325 eggs (0.31%) was positive for *S. enteritidis* when the yolk contents were cultured without the yolk membrane, and 16 of these 325 eggs (4.92%) were positive when the yolk membrane and contents were cultured together (Figure 2). Overall, for both trials combined, *S. enteritidis* was isolated at a 0.49% frequency from yolk contents samples and at a 4.30% frequency from samples that also included yolk membranes. The differences between the frequencies of *S. enteritidis* recovery from the two types of samples were significant ($P < 0.007$) in both trials.

DISCUSSION

The site of deposition of *S. enteritidis* in egg contents and the potential for movement to other sites can determine whether significant multiplication can occur before refrigeration successfully lowers internal egg temperatures to growth-restricting levels. Deposition within egg yolk contents, as illustrated in the present and prior experiments (Bradshaw et al., 1990; Humphrey, 1990; Gast and Holt, 2000a, 2001), can result in rapid multiplication to high levels of bacteria. Penetration across the yolk membrane into the yolk could similarly provide *S. enteritidis* contaminants with access to yolk nutrients. Some previous studies have suggested that bacterial movement from albumen into yolks is uncommon during the first week of incubation (Kim et al., 1989; Humphrey and Whitehead, 1993), but more rapid migration may occur at higher initial bacterial levels and at warmer incubation temperatures (Hammack et al., 1993; Braun and Fehlhaber, 1995; Gast and Holt, 2000a).

Physical and chemical changes in albumen viscosity as eggs age may be necessary to allow migration of *S. enteritidis* from albumen to yolk, but these considerations would not affect inward migration following initial deposition on the yolk membrane itself. In the present study, *S. enteritidis* inoculated onto the yolk membrane penetrated to the yolk contents infrequently at 15 C (even after 72 h of incubation), but did so at a high frequency after 24 h of incubation at 25 C. Moreover, the observed magnitude of multiplication of *S. enteritidis* within egg yolks following penetration from the exterior surface at 25 C was far less than was evident for yolks inoculated internally but was still sufficient by 24 h to represent a significantly heightened threat to a human consumer. The vitelline membrane of freshly laid eggs does not accordingly

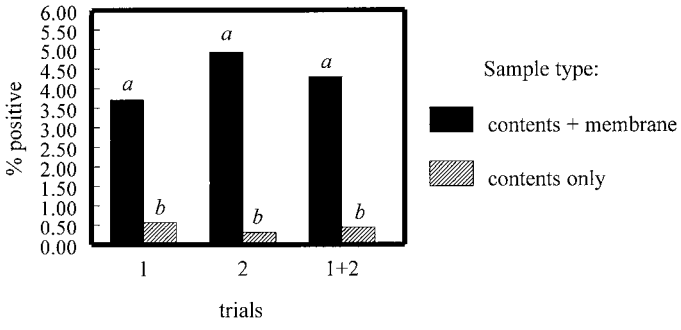

FIGURE 3. Isolation of *Salmonella enteritidis* (SE) from yolks of eggs laid by experimentally infected hens. Two yolk samples from each egg laid between the fourth and twenty-second days postinoculation were cultured to detect SE (n = 350 eggs in Trial 1 and 325 eggs in Trial 2). One sample consisted of ½ of the interior contents of each yolk, and the other consisted of the remaining yolk contents plus the yolk membrane. The SE isolation frequencies from the two types of samples were significantly ($P < 0.05$) different where designated by different lowercase letters.

appear to represent a significant barrier to bacterial movement into the yolk interior, but even a partial degree of success in lowering internal egg temperatures should severely reduce the likelihood that such penetration (and subsequent multiplication) will occur.

The spatial distribution of *S. enteritidis* deposition inside eggs has been a topic of considerable interest for formulating dependable egg refrigeration standards. Because albumen is not a supportive environment for microbial growth, deposition in that location would provide a margin of safety during the interval before eggs are fully cooled. Previous investigations have included instances in which yolk (Humphrey et al., 1989; Timoney et al., 1989; Bichler et al., 1996; Gast and Holt, 2000b) or albumen (Gast and Beard, 1990; Shivaprasad et al., 1990; Humphrey et al., 1991) contamination was most prevalent, although all of these experiments reported the isolation of *S. enteritidis* from both locations. Far less information is available about the distribution of contaminants between interior and exterior yolk locations. In a prior infection study, *S. enteritidis* was isolated from entire yolks but not from very small (1.0-mL) samples of yolk contents (Gast and Beard, 1990). In the present study with samples of yolk contents that were large enough to be more fairly representative, only about 10% of all yolks contaminated with *S. enteritidis* were detected by culturing yolk contents (without yolk membranes). Although extremely rapid penetration into the contents before the eggs were collected and sampled could also account for this observation, these results suggest that initial deposition of *S. enteritidis* inside egg yolks may occur at a low frequency. In most instances, however, it appears that the vitelline membrane is the more common initial site of yolk-associated contamination.

Because *S. enteritidis* is seldom deposited within egg contents, and because penetration of *S. enteritidis* through the yolk membrane occurs so slowly at lower temperatures, prompt refrigeration of eggs should minimize opportunities for rapid bacterial multiplication using egg yolk nutrients. However, the occasional deposition of *S. enteritidis* in or on yolks poses a potentially heightened risk if internal egg temperatures are not lowered rapidly enough. Risk assessment calculations have estimated that approximately 3.2 million eggs contaminated with *S. enteritidis* may be produced annually in the United States (Ebel and Schlosser, 2000). Even if only a very small percentage of these instances involve yolk deposition, tens or hundreds of thousands of eggs might be able to support rapid bacterial growth during the early stages of current cooling practices. Recent innovations in egg cooling technologies, including the use of cryogenic gases, forced air, and modified egg cartons or baskets, offer hope that more rapid temperature control during egg storage may soon be feasible (Curtis et al., 1995; Thompson et al., 2000). Moreover, the possibility that *S. enteritidis* can reach and multiply in egg yolk contents should not undermine confidence in the overall wisdom and efficacy of egg refrigeration but instead should serve as a reminder that risk reduction programs must include a full spectrum of control strategies to compensate for the inherent limitations of refrigeration (or any other single program component).

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